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13. ABSTRACT *(Maximum 200 Words)*

This project is investigating the potential for inhaled uranium oxide aerosols to penetrate the nose-brain barrier, directly enter the central nervous system (CNS), diffusely distribute within the CNS, and result in slowly developing neurotoxic responses. The first year of the project has focused on generating aerosols representative of exposure concentrations and particle sizes that reflect exposure atmospheres potentially encountered in very short-term exposures likely in the Gulf War. The research team has successfully generated aerosols of tantalum oxide, uranium di- and tri-oxides alone and in combination, and of depleted uranium. For each of these aerosols, a group of rats was exposed to atmospheres of approximately 500 mg/m^3 for 15 minutes. These exposures were done in normal animals and in animals with nasal inflammation. Controls with and without inflammation were exposed to clean, filtered air. Data have only begun to be analyzed. Although no tantalum or uranium was observed in brain tissue two hours following exposure to any of the metals, preliminary data suggest an increase of glial fibrillary acidic protein, a marker for astroglia, was observed in the olfactory bulbs of animals exposed to the most soluble of the compounds, uranium trioxide. Although data are very preliminary, a increasing trend corresponding to solubility of compounds is suggested. Glial activation is generally considered in these acute reactions as a protective response to an insult in the surrounding neurons.

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INTRODUCTION

Purpose: The purpose of the overall project is to test the hypothesis that inhaled uranium–containing aerosols will enter the central nervous system (CNS) via olfactory transport, follow neuronal pathways to distal regions of the CNS, and ultimately result in neurodegeneration. The initial study undertaken in Year 1 addresses short-term, high concentration exposures to uranium oxides and depleted uranium oxide. These studies will examine the effects of inhaled uranium oxides both in a healthy rat model and one in which inflammation has been induced in the upper respiratory tract. Target organs to be examined include nasal and lung tissue, brain, and kidney. A one-year time course of response following these short-term, high dose exposures will be determined with sacrifices scheduled on 0, 30, 180, and 360 days post-exposure. Results of these initial exposures will address all of the original hypotheses:

Hypothesis I. Inhalation of uranium aerosols during the Gulf War from combustion of DU containing weapons resulted in CNS deposition and subsequent neurodegeneration in a subset of those exposed.

Hypothesis II: Transient conditions such as inflammation compromised the olfactory epithelium and enhance the entry of uranium and the subsequent development of neurodegeneration

Hypothesis III: Markers of neurodegeneration are correlated with the concentration and deposition of U within the CNS following inhalation exposure.

Hypothesis IV: The degree of and time-course of neurodegeneration are dose and exposure duration dependent.
These hypotheses will be tested for both short- and long-term exposure scenarios as differences related to exposure rate and dose may be critical to uptake, clearance, and ultimate neurotoxicity.

Approved Scope of Work: Year 1

Uranium and tantalum oxide aerosols will be generated, characterized, and tested on the nose-only inhalation exposure system at Lovelace Biomedical and Environmental Research Institute (LBERI). Separate groups of rats will then be exposed via nose-only inhalation for 15 minutes to aerosols of 1)insoluble UO_2; 2)soluble UO_3; 3) a mixture containing by weight 50% UO_2 and 50% UO_3; and 4) TaO_2 (a negative control) at concentrations of 500 mg/cm^3. A fifth group will be co-exposed to endotoxin (to induce inflammation) and the UO_2UO_3 mixture for 15 minutes. Thirty animals will be exposed in each group. Separate air-only and endotoxin-air exposed control groups will be employed as vehicle controls.

Serial sacrifices and analyses of animal tissues from these 15 minute exposures will begin in Year 1. Rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180, and 360 days (Year 2) post exposure. Quantitation of metals in nose and brain tissues will be performed with Atomic Absorption Spectrophotometry (AAS) and microbeam Proton Induced X-ray Emission (μ-PIXE). Immunohistochemistry (IHC) of heat shock proteins (HSP) will be done on nasal and representative brain tissues. Brain tissues will also be examined using IHC for the persistence of tyrosine hydroxylase-containing neurons and for the persistence of NeuN labelling. Neuronal damage will be monitored by GFAP (glial fibrillary acidic protein)-IHC. A marker of neuronal degeneration (Fluorojade histochemistry) and a preliminary indicator of the neuronal apoptosis (TUNEL) will also be examined in sections from brains showing U deposition. If data from any of these categories indicates that there is neurodegeneration, specific brain areas such as the substantia nigra (SN) and anatomically-linked areas will be studied in greater detail using IHC directed against other DA cell markers such as the postsynaptic receptors D1 and D2 within the substantia nigra, caudate putamen and olfactory bulbs. Immunoinflammatory markers (IL-1, IL-6, TNFa and their receptors) and immunohistochemistry to detect 4-hydroxynonelation of nigral dopaminergic neurons will also be performed. One kidney and one lung from each sacrificed animal will be analyzed by AAS for inhaled metal content. The other lung and kidney from each sacrificed animal will undergo histopathological examination. Data from the kidney analyses will allow us a measure of potential nephrotoxicity and will afford a comparison of inhalation data with nephrotoxic effects arising from the study of DU

implants. Data from lung will afford an assessment of any pulmonary damage resulting from the inhalation exposures. Multivariate analyses will be used to examine differences between the groups in both the concentrations of metal localized within different brain regions, and the levels of the indicators of neurodegeneration and other markers analyzed. In conditions where the N is not sufficient for this parametric analysis, nonparametric and qualitative analyses will be utilized instead. Note: Tissue analyses for rats exposed in Year 1 will begin in Year 1, but substantially carry over to Year 2.

Progress on Year One Scope: Short Term, High Concentration Exposure Study

The following section is organized by task identified in the original scope of work documented above. The relevant section of the scope, quoted and in bold italics, begins each description of work completed.

TASK ONE: ***Uranium and tantalum oxide aerosols will be generated, characterized, and tested on the nose-only inhalation exposure system at Lovelace Biomedical and Environmental Research Institute (LBERI).***

Methods and Materials: Task 1

Chemicals:

Uranium Oxides – Purchased from CERAC, Inc. P.O. Box 1178, Milwaukee, WI 53201-1178
UO_2 – CAS# 1344-57-6 50 mesh, 99.8% purity
UO_3 – CAS# 1344-58-7 Powder, 99.8% purity
Tantalum oxide - Purchased from CERAC, Inc. P.O. Box 1178, Milwaukee, WI 53201-1178
Ta_2O_5 – CAS# 1314-61-0 325 mesh, 99.99% purity
Depleted uranium 0.75% Ti oxide – Purchased in crude form from Manufacturing Sciences Corporation, Oak Ridge, TN 37830.

Aerosol Generation

Ordering and receipt of material, milling, aerosol generation, and characterization for all compounds was completed in August 2002. Aerosols of the UO_2, UO_3, and Ta were generated using a Venturi powder disperser (Cheng et al., 1989) and a screw feeder (Model 100, AccuRate, Whitewater, WI). DU oxide was prepared by heating DU 0.75% Ti metal – about 30 mesh, as is, purity not determined. All stock materials required prior ball milling to approximately 5 mm and sieving to achieve a material size suitable for aerosol generation. Aerosols generated by the Venturi powder disperser and screw feeder system were diluted with clean, filtered air and passed through a cyclone to remove the fraction of aerosol larger than approximately 5 microns. The aerosol was then fed into a 96-port nose-only exposure system which was operated at a flow rate of approximately 20 L/min. The screw feeder speed was adjusted to deliver the target concentration to the exposure chamber. These procedures follow Standard Operating Procedures used within LRRI (Dunnick et al., 1988; Raabe et al., 1970). Prior to beginning actual exposures, all aerosols were tested in the exposure system to ensure target concentrations were achievable, could be reliably generated, and maintained for the necessary duration. Also, aerosols were evaluated for particle size and chemical purity. Procedures used in this process are discussed in the following section on aerosol characterization.

Difficulties encountered

Selection and development of an aerosol generation system presented a number of challenges. First, the target concentration of 500 mg/m^3 represents a limiting parameter in selecting a powder generator. Most generators will not reliably operate at these high concentrations. The Venturi powder disperser/screw feeder generation system was selected as the system that had the highest potential of successfully generating 500 mg/m^3 with a respirable size distribution. Once selected, the Venturi powder disperser/screw feeder generation system had to be extensively tested before the high concentrations were attainable. Second, the size distribution of the bulk powders was much too large

to provide a respirable aerosol. This was made more difficult because the densities of the uranium compounds were high, ranging from 7.29 to 19.05, so the real particle size is much smaller than the aerodynamic size. A ball mill was purchased and used to mill materials, but it required significant milling time before an acceptable fraction of the materials were in the respirable size fraction. Third, the per cent of material in the respirable size range (less than 5 microns) for each compound was substantially less than originally anticipated. As a result, each powder was first ball milled and then sieved with 400 mesh screen [37 micron pore size]. A very small fraction of each powder was recovered from the milling and was used to load the screw feeder. Because of the small usable fraction, additional material had to be ordered and the milling repeated. Per cent recovery for this process varied across the compounds. The lower than anticipated yield of suitable material from the UO_3 stock also resulted in substantial delays due to inability of the supplier to meet the demand. DU was not included in the original protocol because of uncertainties in characterization, supply, and purity of available source materials. Our original thoughts were that scientifically examination of the well characterized forms of uranium would yield a better understanding of mechanisms underlying observed responses and allow for extrapolation of the results to the more likely battlefield exposures to DU. However, in planning the short-term exposures we were able to identify a supplier and a method of processing that would allow us to examine this more environmentally relevant material in parallel to our other studies in a cost-effective manner. We have therefore added this exposure condition for completeness and to reduce the uncertainties introduced by extrapolation. However, the crude form of the material resulted in the lowest percentage yield of suitable particle size.

Exposure Atmosphere Characterization

As mentioned above, characterization of the exposure atmospheres included determining the aerosol concentration, particle size distribution and chemical determination. Zefluor filter samples (1.0 micron pore size, 25mm diam. Gelman Sciences, Ann Arbor MI) were taken at two locations simultaneously, one from each side of the chamber. A nominal flow of 2 LPM was drawn through each filter. Actual flow rates were measured with rotameters. Aerosol concentration for the sampling period was determined by dividing the total mass collected on the filter by the airflow through the filter. The aerosol particle size distributions were determined using a Lovelace multi-jet cascade impactor (InTox Products, Albuquerque, NM). Aerosol samples were collected a minimum of two times during each exposure. The MMAD and geometric standard deviation were calculated using an LRRI computer code. Target particle size of 2 um MMAD ensured a high deposition in the nose. The chemical characterization was determined on the filter samples using AAS analysis in the laboratory of Graham Bench (Lawrence Livermore National Laboratory [LLNL]). The analyses determined the purity of the exposure compounds.

Environmental Conditions of the Exposure Chamber

Temperature was monitored before and after exposure and held between 18 and 22° C. Oxygen concentration was monitored at the same time and maintained above 18%.

Results: Task 1

Aerosol Exposures

Overall, the results for aerosol exposures were satisfactory. The exposures with UO_3 and UO_2+UO_3 were significantly below target concentration of 500 mg/m^3 due to difficulties in generating the UO_3 powder. However, these data are still considered valid for the test of high concentration-acute exposures in these studies. Actual exposure concentrations are summarized in Table 1.

Table 1. Summary of Exposure Aerosols

CHEMICAL COMPOUND	*MEAN* CONCENTRATION (mg/m^3)	*PARTICLE* SIZE (MMAD)	*Sigma-g*	*PERCENT* OF TARGET*
TaO_5	548.01	2.13	1.92	109.60
UO_3	328.75	1.56	1.71	65.75
DU	608.81	2.02	1.36	121.76
UO_2	572.05	2.38	1.39	114.41
$UO_2 + UO_3$	304.66	1.97	1.52	60.93

*Target concentration for all exposure groups was 500 mg/m^3.

TASK TWO: ***Separate groups of rats will then be exposed via nose-only inhalation for 15 minutes to aerosols of 1)insoluble UO_2; 2)soluble UO_3; 3) a mixture containing by weight 50% UO_2 and 50% UO_3; and 4) TaO_2 (a negative control) at concentrations of 500 mg/cm^3. A fifth group will be co-exposed to endotoxin (to induce inflammation) and the UO_2UO_3 mixture for 15 minutes. Thirty animals will be exposed in each group. Separate air-only and endotoxin-air exposed control groups will be employed as vehicle controls.***

Serial sacrifices and analyses of animal tissues from these 15 minute exposures will begin in Year 1. Rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180, and 360 days (Year2) post exposure.

Methods and Materials: Task 2

Animals and Animal Husbandry

Animal Purchase and Housing. A total of two hundred ninety (145 male and 145 female) Fischer 344 rats, 9-10 weeks old, (Harlan Sprague Dawley, Indianapolis, IN) were used. All rats were quarantined for 10 days, housed 2 to 3 per cage in shoebox cages with hardwood chip bedding. They were fed Teklad Certified Rodent Diet (W). Food and water were available *ad libitum* except during exposure. The animal rooms were maintained at 20-22°C and 30-70 % relative humidity. A 12-hour, light/dark cycle, was maintained with lights on at 0600. Before exposure, the rats will be conditioned to nose-only restraint tubes for at least two periods. The first will be for about 20 minutes, the second, conducted on a separate day, will be for about 40 minutes.

Animal Randomization and Identification. The rats were randomized by weight into exposure groups of 20 or 30 rats each, equal numbers of males and females (Tables 2 and 3). Rats were identified by tail tattoo using an alpha numeric numbering system.

Animal Selection and Justification for Use. Previous work in rats with inhalation of metal aerosols has shown that several metals will pass through the olfactory epithelium into the olfactory bulb of the brain (Lewis and Dahl, 1994, Divine et al., 1999, Brennerman et al 2000, Sunderman, 2001). In addition, some metals (e.g. manganese, nickel, zinc) can cross synapses in the olfactory bulb and migrate to distant nuclei of the brain. This work indicates that the rat is a good species to test the hypothesis that the same phenomenon occurs with inhaled uranium oxide aerosols.

Endotoxin Instillation

To induce inflammation in the nasal mucosa, four groups of rats (Table 3) were intranasally instilled with endotoxin (Sigma Chemical Co., St. Louis, MO, Lipopolysaccharide from Pseudomonas aerugenosa Serotype 10, 1 mg/ml) following the procedures of Harkema and Hotchkiss (1991). Forty-eight hours before exposure to particulate aerosols or air, rats were anesthetized by halothane inhalation, removed in a light plane of anesthesia, and instilled with endotoxin (200 μg total) by placing 2 - 50 μl drops in each nostril.

Table 2. Uranium Entry to CNS – Exposures and Scheduled Sacrifices

Exposure Group & Exper. #	Exposure Compound	Exposure Conc.* (mg/m3)	Total # Rats Exposed	*0 d sac*	*30 d sac*	180 d sac	360 d sac	Spare Rats
1A – 7208	Air**	0	10m/10f	***2m/2f***	***2m/2f***	2m/2f	2m/2f	2m/2f
1B – 7209	UO2	500 mg/m3	15m/15f	***3m/3f***	***3m/3f***	3m/3f	3m/3f	3m/3f
1C – 7210	UO3	500 mg/m3	15m/15f	***3m/3f***	***3m/3f***	3m/3f	3m/3f	3m/3f
1D – 7211	UO2+UO3	500 mg/m3	15m/15f	***3m/3f***	***3m/3f***	3m/3f	3m/3f	3m/3f
1E – 7212	TaO2	500 mg/m3	15m/15f	***3m/3f***	***3m/3f***	3m/3f	3m/3f	3m/3f
1F - 7213	DUO_x**	500 mg/m3	15m/15f	***3m/3f***	***3m/3f***	3m/3f/	3m/3f/	3m/3f
	Total # rats		170	***34***	***34***	34	34	34

* All exposures 15 minutes duration ** = 2 hours after end of exposure

NOTE: Those in bold italics have been completed to date

Table 3. Inflammation and U Entry to CNS – Exposures and Scheduled Sacrifices

Exposure Group & Exper. #	Exposure Compound	Exposure Conc.* (mg/m3)	Total # Rats Exposed	*0 d** sac*	*30 d sac*	180 d sac	360 d sac	Spare Rats
2A – 7214	Air	0	10m/10f	***2m/2f***	***2m/2f***	2m/2f	2m/2f	2m/2f
2B –	UO2+UO3	500	Same as exposure group 1D					
2C – 7215	UO2+UO3+ Endotoxin	500	15m/15f	***3m/3f***	***3m/3f***	3m/3f	3m/3f	3m/3f
2D - 7216	Endotoxin	500	15m/15f	***3m/3f***	***3m/3f***	3m/3f/	3m/3f/	3m/3f
2E - 7217	DUO_x + Endotoxin	500	15m/15f	***3m/3f***	***3m/3f***	3m/3f	3m/3f	3m/3f
	Total # rats		110	22	22	22	22	22

* All exposures 15 minutes duration ** = 2 hours after end of exposure

NOTE: Those in bold italics have been completed to date.

Inhalation Exposures

Groups of rats, with and without prior endotoxin instillation, were exposed nose-only for 15 minutes to 500 mg/m3 of UO2, UO3, DUO, the mixture of UO2/UO3 or to air only (control group) as outlined in Table 2. Aerosols were generated from a Venturi powder disperser into a 96 port nose-only chambers previously tested to verify exposure atmospheres as described above.

Observations and Measurements at Scheduled or Moribund Sacrifice

Body Weight and Clinical Observations

Rats were observed twice daily for morbidity and mortality. Body weights were obtained within one week of the beginning of exposures for the purpose of randomization. Body weights were obtained the

day before exposures began and monthly thereafter. Detailed clinical observations are made at the time of weighing.

Necropsy

Necropsies are performed on all rats dying or sacrificed in a moribund condition. Organs of the cranial, thoracic and abdominal cavities are examined for lesions and the tissues listed in Table 3 are fixed for potential future examination.

A specialized examination is performed on rats at the time of scheduled sacrifice. The necropsy procedures are the same for all groups. The organs and tissues sampled and their preservation is shown in Table 4.

The rats are sacrificed by exposure to CO_2 and exsanguination by cardiac saline perfusion. A body weight is be obtained. The brain is removed first, weighed and frozen in liquid-nitrogen-cooled isopentane at –36o C and transferred at the end of the day to –80° C for long term storage until subsequent serial cryosection. Quantitative PIXE analysis, IHC and qualitative pathology are performed on the brain.

The nose, with skin and lower jaw removed is fixed in 4% paraformaldehyde. IHC and qualitative pathology are performed on the nose at UNM.

The left and right lungs are weighed. The left lung is perfused with 4% paraformaldehyde and the right is frozen. Qualitative pathology is done on the left lung at LRRI and AAS on the right lung at LLNL.

The larynx, trachea and bronchial lymph node are fixed in 4% paraformaldehyde for histopathology at LRRI.

The left and right kidneys are weighed. The left kidney is fixed in 4% paraformaldehyde and the right is frozen. Histopathology is done at LRRI and AAS analysis at LLNL.

Both femurs are weighed and frozen for chemical analysis.

Table 4. Organs Sampled and Preservation Method

Tissue	Weight	Preservative	Histo-pathology	Chemistry	Analysis By
Brain	X	Freeze –36°C Hold - 80°C	X	X	UNM/LLNL
Nose		4% Paraform.	X		UNM
Lung - left	X	4% Paraform	X		LRRI
Lung – right	X	Freeze – 20		X	LLNL
Larynx		4% Paraform	X		LRRI
Trachea		4% Paraform	X		LRRI
LN, Bronchial		4% Paraform	X		LRRI
Kidney – left	X	4%Paraform	X		LRRI
Kidney – right	X	Freeze -20		X	LLNL
Femurs - both	X	Freeze - 20		X	LLNL

Preparation of tissues for quantitation of metal deposition at LLNL

The lung, kidney and femur samples were prepared for analysis by dry ashing at 550°C at LRRI. The ashed samples were sent to LLNL for quantitative AAS of kidney, lung and femur. Frozen brains were

transferred to UNM for cryosectioning prior to transfer to LLNL for PIXE analyses using in-house protocols and SOPs.

Histopathologic examination of selected tissues at LRRI

Samples of larynx, trachea, lung, bronchial lymph node and kidney were examined for lesions by light microscopy. Formalin-fixed tissues samples were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin.

The microscopic findings in the lung were graded using the criteria show in Table 5.

Table 5. Histologic Criteria for Grading Severity of Lung Lesions

Diagnosis	Grade	Criteria
Particle loading of alveolar macrophages	NAD	Essentially no particles in scant cytoplasm
	Minimal	A few black particles scattered in cytoplasm or a few macrophages with abundant particles
	Mild	Moderate number of particles in cytoplasm (≤10); do not obscure nucleus of macrophage
	Moderate	Many particles (too many to count) in cytoplasm cover the nucleus; slightly enlarged cytoplasm
Alveolar macrophage hyperplasia	NAD	Few scattered AM in alveoli; difficult to find
	Minimal	Minimal increase in number of AM
	Mild	Mild increase in number of AM; easily found at high magnification; average 1/alveolus
	Moderate	Moderate increase in number of AM; easily found at low magnification; several macrophages/alveolus
Broncho-Intersticial pneumonia	NAD	No inflammation present
	Minimal	A few inflammatory cells infiltrating septa around bronchioles; involves < 10% of lung
	Mild	Infiltrating inflammatory cells involving 10-25% of the lung, may also be in alveoli
	Moderate	Infiltrating inflammatory cells involving 26-50% of the lung, also in alveoli
	Marked	Infiltrating inflammatory cells involving > 50% of lung, also in alveoli
Uremic pneumonia	NAD	Edema and a few PMN in alveoli, involves < 10% of lung
	Mild	Edema and PMN in alveoli, Ca present, involves 10-25% of lung
	Moderate	Edema and PMN in alveoli, Ca present, involves 26-50% of lung
	Marked	Edema and PMN in alveoli, Ca present, involves > 50% of lung

NAD = No abnormalities detected

Grading of kidney pathology

The microscopic findings in the kidney were graded using the following criteria. Renal tubular necrosis is a sequelae of uranium toxicity. It is characterized by necrosis of the tubular epithelium in tubules of the pars recta portion of the proximal tubules of the kidney. The epithelium is sloughed into the lumen of the tubule. With time the epithelium regenerates.

NAD – No evidence of tubular necrosis
1 - Few, scattered foci of tubular necrosis, slough of tubular epithelial cells
2 – Mild tubular necrosis in pars recta, sloughing of cells, hyaline casts, involves 5- 25% of the proximal tubules
3 – Moderate, tubular necrosis in pars recta, sloughing of cells, hyaline casts, involves 25-50% of the proximal tubules
4 – Marked, tubular necrosis in the pars recta, sloughing of cells, hyaline casts, mineralization, involves > 50 % of the proximal tubules

A spontaneous ageing renal disease, nephropathy, is characterized by progressive involvement of glomeruli and tubules, with thickening of basement membranes, focal tubular regeneration, mononuclear inflammatory cell infiltrates, glomerular hyalinization and sclerosis, tubular hyaline casts, interstitial fibrosis, mineralization and cyst formation. Grading was based on greater severity of the lesions comprising the nephropathy and the extent of involvement.

NAD – No evidence of nephropathy
1 - Few, scattered foci of tubular regeneration with thickened basement membrane and karyomegaly
2 – Mild tubular regeneration, hyaline casts and cellular infiltrates, involves < 25% of the cortical parenchyma
3 – Moderate, tubular and glomerular fibrosis, involves 25-50% of the cortical parenchyma
4 – Marked, tubular and glomerular fibrosis, involves > 50 % of the cortical parenchyma

Results: Task 2

All animals identified in Tables 2 and 3 have been exposed, and 0 and 30 day sacrifices have been completed as noted in those tables.

Sacrifice Schedule

The exposures were conducted the week of August 5, 2002. The sacrifice schedule is noted in Table 6 A and B. As in Tables 2 and 3, bold italics note sacrifices completed to date.

Table 6. Sacrifice Scheduled Dates

A. Uranium Entry to CNS – Experiment #'s and Range #'s

Exposure Group	Exposure Material	Experiment Number M/F	*Range of Numbers* M/F	*0 d sac*	*30 d sac*	180 d sac	360 d sac
1A –	**Air****	7208 / 7209	A001-010 / 011-020	***8/5/02***	***9/4/02***	2/3/03	8/4/03
1B –	**UO2**	7210 / 7211	B001-016 / 017-	***8/8/02***	***9/5/02***	2/5/03	8/7/03
1C –	**UO3**	7212 / 7213	C001-016 / 017-	***8/6/02***	***9/3/02***	2/4/03	8/5/03
1D –	**UO2+UO3**	7214 / 7215	D001-016 / 017-	***8/9/02***	***9/6/02***	2/7/03	8/4/03
1E –	**TaO2**	7216 / 7217	E001-016 / 017-	***8/5/02***	***9/3/02***	2/3/03	8/4/03
1F -	**DUO_x ****	7218 / 7219	F001-016 / 017-	***8/7/02***	***9/4/02***	2/5/02	8/6/03
	Total # rats		**180**	***34***	***34***	**34**	**44**

B. Inflammation and U Entry to CNS – Experiment #'s and Range #'s

Exposure Group	Exposure Material	Experiment Number M/F	Range of Numbers M/F	*0 d** sac*	*30 d sac*	180 d sac	360 d sac
2G –	**Air**	7220 / 7221	G001-010 / 011-020	***8/6/02***	***9/3/02***	2/4/03	8/5/03
2H –	**UO2+UO3**	Same as Exposure Group 1D					
2I –	**UO2+UO3 + Endotoxin**	7222 / 7223	I001-015 / 016-030	***8/9/02***	***9/6/02***	2/7/03	8/8/03
2J -	**Endotoxin**	7224 / 7225	J001-015 /016-030	***8/8/02***	***9/5/02***	2/6/03	8/7/03
2K -	**DUO_x + Endotoxin**	7226 / 7227	K001-015 / 016-030	***8/7/02***	***9/4/02***	2/5/03	8/6/03
	Total # rats		**110**	***22***	***22***	**22**	**22**

Pathology completed to date – Planned and Moribund Sacrifices

Early Deaths – Fifteen rats died or were sacrificed moribund 2 to 13 days after inhalation exposure. (Table 7) All fifteen had been exposed to UO_3, the most soluble of the compounds used. Twelve of the fifteen were female. One rat died 2 days after inhalation exposures with no obvious lesions and the cause of death was undetermined. The remaining 14 had acute tubular necrosis in the pars recta portion of the proximal tubules. Those that survived longer had some tubular epithelial regeneration. In addition, all rats with tubular necrosis had uremic pneumonia, and in 12 of the 14 the pneumonia was moderate to marked. The pulmonary lesion was characterized by infiltration of neutrophilic inflammatory cells in the septa and alveoli, edema and calcium precipitates.

The acute tubular necrosis and the uremic pneumonia are consistent with a uranium-induced renal toxicity resulting in uremia and pneumonia actually causing death or illness of the animals. (Haley 1982)

Zero day sacrifices – Fifty-six rats were sacrificed two hours after the end of inhalation exposure, as scheduled. Only minimal histologic changes were evident in the lung of the exposed rats.(Table 8) A few small dark round particles could be seen in the cytoplasm of alveolar macrophages of some of the rats exposed to the more insoluble compounds, UO2, TaO2 and DUOx. A minimal increase in the number of alveolar macrophages was noted in most of the rats exposed to particles.

A broncho-interstitial pneumonia was present in six of the 18 rats intranasally instilled with endotoxin two days before sacrifice. The pneumonia was moderate to marked in four of the six rats indicating involvement of more that 25% of the lung. The pneumonia was undoubtedly a reaction to endotoxin that passed from the nasal cavity to the lung. (Harkema and Hotchkiss 1992).

30-day sacrifices – These sacrifices were competed the week of September 3, 2002. Because of the early death of rats in the UO3 group, only 1 female remained for the 30 day sacrifice. Tissue sections have been prepared on all the sacrificed rats and will be reviewed the first part of FY03.

Table 7. Histologic Lesion Summary – Early Deaths and Moribund Sacrifices

Exper #	An #	Death Type / DPE	Larynx	Trachea	BLN Histeo-cytosis	Kidney Nephro-pathy	Lung Tubular Necrosis	Lung Uremic Pneumonia	Lung AM Hyper.
7212	C007	ND/8	—	M	M	—	4	3 acute	—
	C008	ND/13	M	—	M	—	3	1 acute	—
	C010	ND/2	—	—	—	—	—	—	—
7213	C017	ND/10	—	M	M	—	3	3 chronic	—
	C018	ND/6	M	—	2	—	4	4 acute	—
	C019	MSAC/7	—	—	M	—	3	4 chronic	—
	C020	ND/6	—	—	M	—	3	3 chronic	—
	C021	MSAC/7	—	—	2	—	3	4 chronic	—
	C022	ND/6	—	—	M	—	4	4 acute	—
	C023	ND/6	—	—	M	—	4	3 chronic	—
	C024	ND/4	—	—	1	—	4	2 acute	—
	C025	ND/6	—	—	2	—	4	3 acute	—
	C027	ND/8	—	—	M	—	4	4 acute	—
	C028	ND/7	—	—	M	—	4	4 acute	—
	C029	ND/8	—	—	1	—	4	4 acute	—

DPE = Days Post Exposure ND = Natural Death MSAC = Moribund Sacrifice M = Missing

Table 8. Histologic Lesion Summary – Four Hour Post Exposure Sacrifice

						Kidney	Lung		
Exper #	Animal #	Exposure Compound	Larynx	Trachea	BLN	Nephro-pathy	AM Particles	AM Hyper	Broncho Interstitial Pneumonia
7208	A009	AIR	—	—	—	—	—	—	—
	A010	AIR	—	—	M	—	—	—	—
7209	A019	AIR	—	—	—	—	—	—	—
	A020	AIR	—	—	M	—	—	—	—
7210	B014	UO_2	—	—	—	—	2	2	—
	B015	UO_2	—	—	—	1	1	1	—
	B016	UO_2	—	—	M	—	1	—	—
7211	B030	UO_2	—	—	—	—	—	1	—
	B031	UO_2	—	—	—	—	1	—	—
	B032	UO_2	—	—	—	—	1	1	—
7212	C014	UO_3	—	—	M	1	—	1	—
	C015	UO_3	—	—	—	1	—	1	—
	C016	UO_3	—	—	—	—	—	1	—
7213	C030	UO_3	M	—	M	—	—	—	—
	C031	UO_3	—	—	—	—	—	1	—
	C032	UO_3	—	—	—	—	—	1	—
7214	D014	$UO_2 + UO_3$	M	—	—	—	—	1	—
	D015	$UO_2 + UO_3$	—	—	—	—	—	1	—
	D016	$UO_2 + UO_3$	—	—	M	—	—	1	—
7215	D030	$UO_2 + UO_3$	—	—	—	—	1	2	—
	D031	$UO_2 + UO_3$	—	—	—	—	1	1	—
	D032	$UO_2 + UO_3$	—	—	M	—	—	1	—
7216	E014	TaO_2	—	particles	M	—	—	—	—
	E015	TaO_2	—	—	M	1	—	—	—
	E016	TaO_2	—	—	M	—	1	—	—
7217	E030	TaO_2	—	M	M	—	1	—	—
	E031	TaO_2	M	—	M	—	1	1	—
	E032	TaO_2	M	—	M	—	1	1	—
7218	F014	DUO_x	—	—	—	—	1	1	—
	F015	DUO_x	—	—	—	1	—	—	—
	F016	DUO_x	—	—	—	—	—	—	—

Table 8. Histologic Lesion Summary – Four Hour Post Exposure Sacrifice (Concluded)

						Kidney	Lung		
Exper #	Animal #	Exposure Compound	Larynx	Trachea	BLN	Nephro-pathy	AM Particles	AM Hyper	Broncho Interstitial Pneumonia
7219	F030	DUO_x	—	—	M	—	—	—	—
	F031	DUO_x	—	—	M	—	1	1	—
	F032	DUO_x	—	—	—	1	1	1	—
7220	G009	AIR	—	—	—	—	—	—	—
	G010	AIR	—	—	—	—	—	—	—
7221	G019	AIR	—	—	—	—	—	—	—
	G020	AIR	—	—	M	—	—	1	—
7222	I013	UO_2 + UO_3 + ENDO	—	—	—	—	1	—	—
	I014	UO_2 +UO_3 + ENDO	—	—	M	—	—	1	—
	I015	UO_2 + UO_3 + ENDO	—	—	—	1	1	—	—
7223	I028	UO_2 + UO_3 + ENDO	—	—	—	—	1	2	3
	I029	UO_2 + UO_3 + ENDO	—	—	—	—	—	1	2
	I030	UO_2 + UO_3 + ENDO	—	—	—	—	—	1	3
7224	J013	ENDOTOX	—	—	—	—	—	1	—
	J014	ENDOTOX	—	—	—	1	—	1	2
	J015	ENDOTOX	—	—	—	1	—	1	—
7225	J028	ENDOTOX	—	—	—	—	—	1	4
	J029	ENDOTOX	—	—	—	—	—	—	—
	J030	ENDOTOX	—	—	—	—	—	1	3
7226	K013	DUO_x + ENDOTOX	—	—	—	—	—	1	—
	K014	DUO_x + ENDOTOX	—	—	—	—	—	1	—
	K015	DUO_x + ENDOTOX	—	—	—	—	—	1	—
7227	K028	DUO_x + ENDOTOX	—	—	—	—	1	1	—
	K029	DUO_x + ENDOTOX	—	—	M	—	1	1	—
	K030	DUO_x + ENDOTOX	—	—	M	—	1	1	—

M = Missing

TASK 3: *Quantitation of metals in nose and brain tissues will be performed with Atomic Absorption Spectrophotometry (AAS) and microbeam Proton Induced X-ray Emission (μ-PIXE).*

Materials and Methods: Task 3

As only the PIXE analysis of 0 day sacrifice brain tissues has been completed to date, only the PIXE methods will be discussed.

Tissue preparation

Frozen tissue sections were cut at 10 microns using a Hacker-Bright motor-driven cryostat. Three levels of sagittal sections containing the brain regions of interest were sampled. These areas were chosen to allow analysis of the primary projection regions of receptor neurons located within the nose (olfactory glomeruli and the spinal nucleus of the trigeminal), and projection regions for the olfactory system and nuclei within the dopaminergic system. One section was mounted on a nylon foil for PIXE analysis. Eight sections from the same level were mounted on Fisher ProbeOn Plus glass slides and retained for immunohistochemical analysis. Anatomical regions within each of the levels are summarized in Table 9.

Table 9. Brain regions within each sampled sagital level of rat brain.

Level of Sectioning	Lateral distance from midline	Anatomic Structure Sampled
1	~ 4 mm	Caudate putamen Spinal nucleus
2	~3 mm	Substantia nigra
3	~2 mm	Olfactory bulb Substantia nigra

Serial sections of those sent to LLNL for PIXE analysis were stained with hematoxylin and eosin, digitally scanned (Figure 1A), and regions for PIXE analysis were outlined to ensure beam localization within the correct brain regions (Figure 1B).

PIXE Methods

Uranium and Ta concentrations in localized brains regions were determined with Proton Induced X-ray emission (PIXE). PIXE is an x-ray fluorescence technique that uses MeV energy proton beams to interrogate specimens. It provides accurate quantitation, simultaneous multi- element detection and is capable of micron-scale spatial resolution whilst maintaining down to 1 mg/g elemental sensitivity. Regions of interest within the freeze dried tissue sections were identified visually using stained neighboring serial sections. Regions of interest within samples were irradiated with 3 MeV proton microbeams for doses of up to 15 micro coulombs. Beam spot sizes were typically between 0.3 x0.3 and 0.5 x 0.5 mm. X-ray yields for U and Ta were monitored using an energy dispersive x-ray detector. Yields were converted to quantitative concentrations using thin film standards of Ta and U of known thickness to determine the detector efficiency. The system has been tested on certified standards and has a quantitative accuracy of better than 95% for analysis of metals in biological matrices.

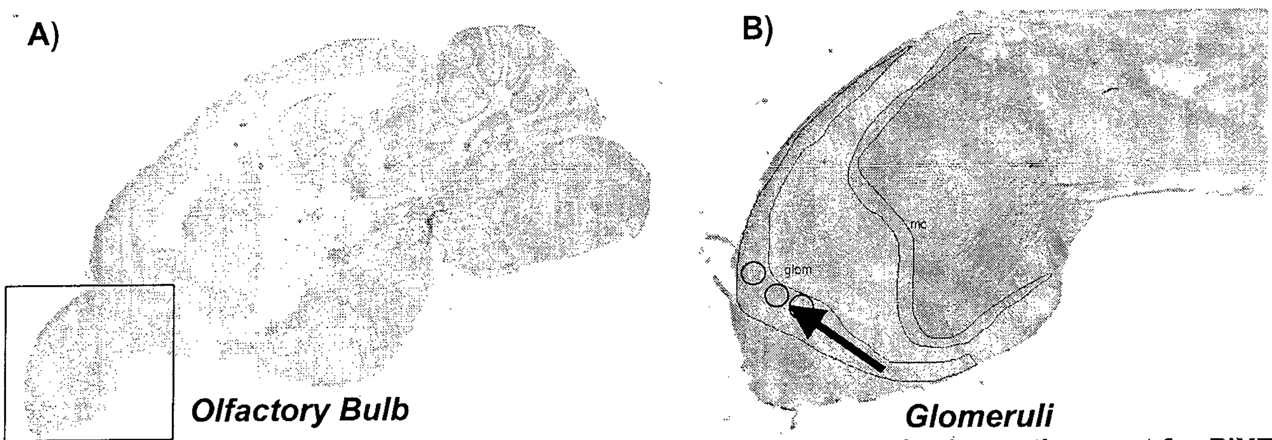

Figure 1. A) Digitally scanned photomicrograph of serial sagittal brain section sent for PIXE analysis. Olfactory bulb shown in inset. B) Magnification of inset in A showing olfactory bulb with glomeruli and mitral cell layer outlined to guide probe alignment for PIXE analysis.

Results: Task 3

No detectable concentrations of metals were observed in either region of the olfactory bulb analyzed following the 0 day sacrifice. Glomeruli and mitral cell layer for all exposure metals and all groups did not exceed the minimum detection limit (MDL). MDLs for each condition are summarized in Table 10.

Table 10. MDL for Uranium and TaO_2 analysis in brain tissues.

				Uranium			***TaO2***	
Study*	**Feature**	**Exposure**	**N**	**mg/kg**	**SD**		**mg/kg**	**SD**
1	Glomeruli	Air**	4	3.6	0.6		2.6	0.3
1		DUOx	6	2.9	0.4			
1		U02	6	2.5	0.2			
1		U02+U03	6	2.5	0.2			
1		U03	6	2.4	0.4			
		TaO2	6				2.2	0.5
1	Mitral	Air**	4	3.6	0.1		2.7	0.4
1		DUOx	6	2.8	0.3			
1		U02	6	2.5	0.3			
1		U02+U03	6	2.7	0.2			
1		U03	6	2.6	0.2			
1		TaO2	6				2.3	0.3
2	Glomeruli	Air + endotoxin	4	3.1	0.3		2.6	0.4
2		DUOx+Endotoxin	6	2.4	0.3			
2		Endotoxin	6	2.6	0.4		2.6	0.3
2		UO2+UO3+Endotoxin	6	2.7	0.3			2.0
2	Mitral	Air + endotoxin	4	3.2	0.5		2.2	0.3
2		DUOx+Endotoxin	6	2.5	0.3			
2		Endotoxin	6	2.4	0.3		2.7	0.3
2		UO2+UO3+Endotoxin	6	2.9	0.2			

- Study 1 refers to exposures without prior endotoxin instillation.
  Study 2 summarizes results with endotoxin

Tissues from the 30 day survival group are currently being analyzed, but results are not yet available.

TASK 4: *Immunohistochemical analysis of brain and nasal tissues will begin in year 1, but extend into year 2. To date, GFAP analysis has been partially completed on a subset of the 0 day sacrifice animals. Those preliminary results will be discussed.*

Because no elevation beyond MDLs was observed in any of the tissues, all immunohistochemical analyses on the 0 day sacrifice animals was restricted to the olfactory bulb glomeruli. This region should be the brain region to first see any uranium or tantalum entering via the olfactory receptor neurons. To date, only staining of level 3 for glial fibrillary acidic protein (GFAP) has been completed in some of the exposure groups for the 0 day sacrifice animals. Only those data will be considered below.

Materials and Methods: Task 4

Sections from level 3 containing the olfactory bulb were reacted with antibody to GFAP (Rabbit anti-cow GFAP-[DAKO Corporation, Denmark]) at a 1:500 dilution. Negative controls were run with each batch of slides, and staining density within 3% of the mean of the negative controls was considered acceptable. Less than 0.4% inter-batch variability in density of background was actually observed across the range of negative control sections. Cy3-conjugated AffiniPure donkey-anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as the secondary fluorescent-tagged antibody. Density of GFAP in stained sections was determined from digital photomicrographs of the fluorescent images. Exposure time was kept constant. Air-exposed control animals were stained with each batch of experimental tissues.

The sampling and analysis procedure is graphically summarized in Figure 2. Sections for analysis were cut from images using a standard 200 x 200 pixel ellipse mask in Adobe Photoshop (Version 7.0) software. These cut samples were then transferred into ImageTool (Version 5, UTHSCSA) software for densitometric analysis. Ellipse samples were pasted within a new image stack to undergo threshold processing to convert the grayscale image to true black and white. The threshold window for all slides was 100 to 255 to enable visualization of neurons and dendrites and maintain consistency across slides and batches (see Figure 2). The number and percent of black and white pixels was then tabulated via an ImageTool analysis function. Three separate sections were cut from each image and analyzed to calculate a mean value for number and percent of black pixels, the indicator for total amount of electrofluorescence staining.

The three black density values for each image are then averaged to compute a mean density value for that animal. Mean values for male, female, and combined group are determined for density and percent black in the image.

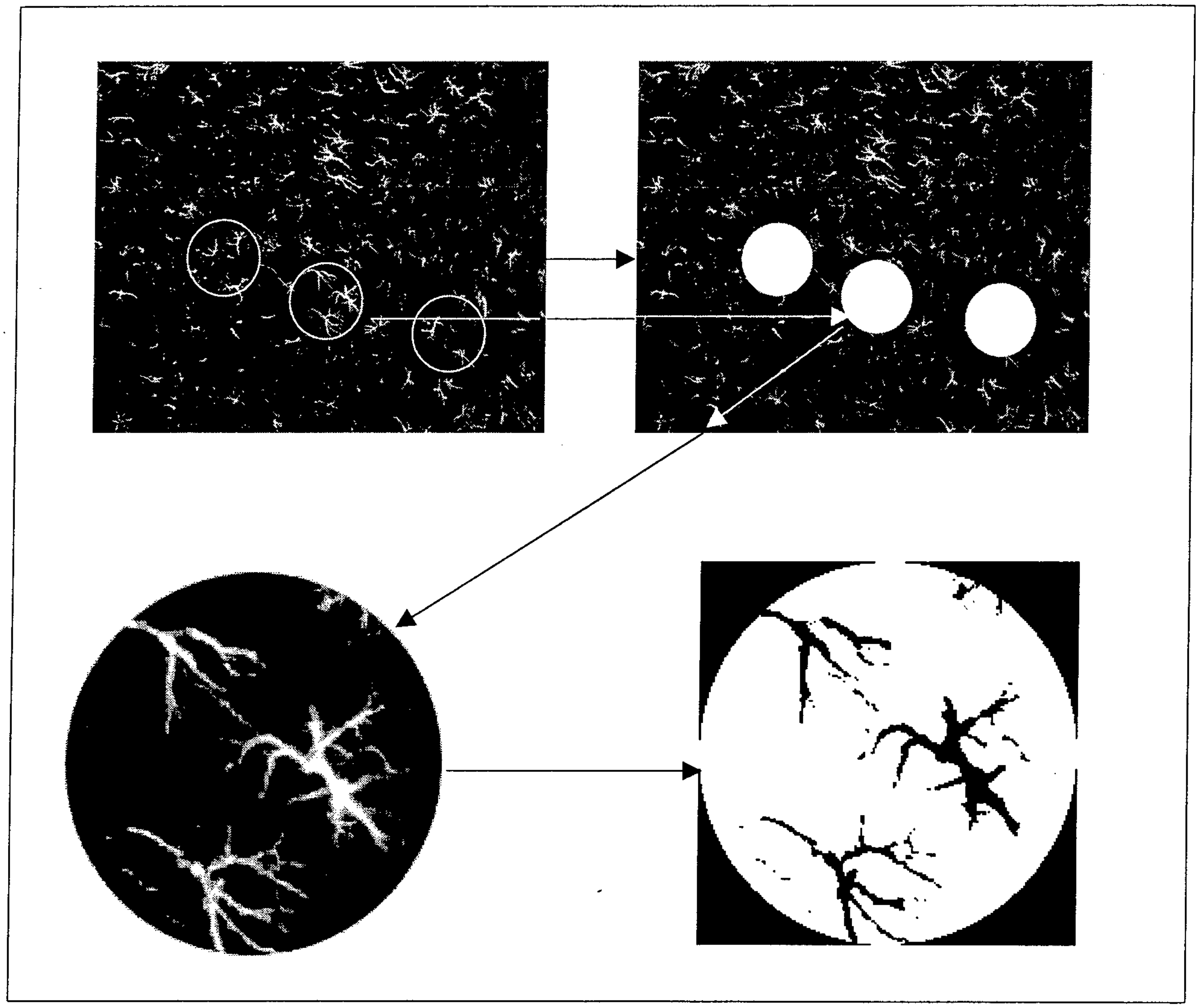

Figure 2. Schematic representation of densitometric analysis procedure. Electrofluorescent image of section of olfactory bulb containing glomeruli as outlined in Figure 1A is photographed (A). Three samples of glomerular staining with anti-GFAP are selected and exported to Image Tool software where images are converted from gray scale to true black and white. Density of black in resultant images is measured and averaged for each structure in each section as a representation of staining intensity. Percent black is likewise determined and an average for each measure for each animal is determined

Results: Task 5

Preliminary analyses have been completed for GFAP density within glomeruli of animals exposed to Air, UO_3, Air + Endotoxin, or UO_3+UO_2+endotoxin. Results show the greatest density of GFAP staining in animals exposed to the most soluble form of uranium, UO_3. While in-depth statistical analyses are premature on these preliminary data, a preliminary analysis of variance across these four treatment groups was significant for treatment ($p < 0.001$), and that a different pattern of responses occurred for females and males (significant treatment by gender interaction, $p < 0.02$). Exploratory analyses of preliminary data using all pair-wise comparisons showed that air-exposed animals did not differ based on prior exposure to endotoxin. However, both the UO_2+UO_3 +endotoxin and the UO_3 group alone differed from each other and from both of the air groups. A significant difference for gender was observed only with UO_3 exposure ($p < 0.001$) when a least-squared means contrast was tested. The probability of the trend across solubility of uranium exposure aerosols, based on the very limited data analyzed to date, was 0.001 when data are pooled across males and females and accounts for 60% of the variance. When trend is examined relative to gender differences, the probability of the observed trend by solubility for gender is 0.07, but accounts for 67% of the variance, consistent with the trend results for pooled data and the sex x treatment interaction observed in the analysis of variance. Data are presented graphically in Figure 3. Bars represent mean values by gender with standard error indicated.

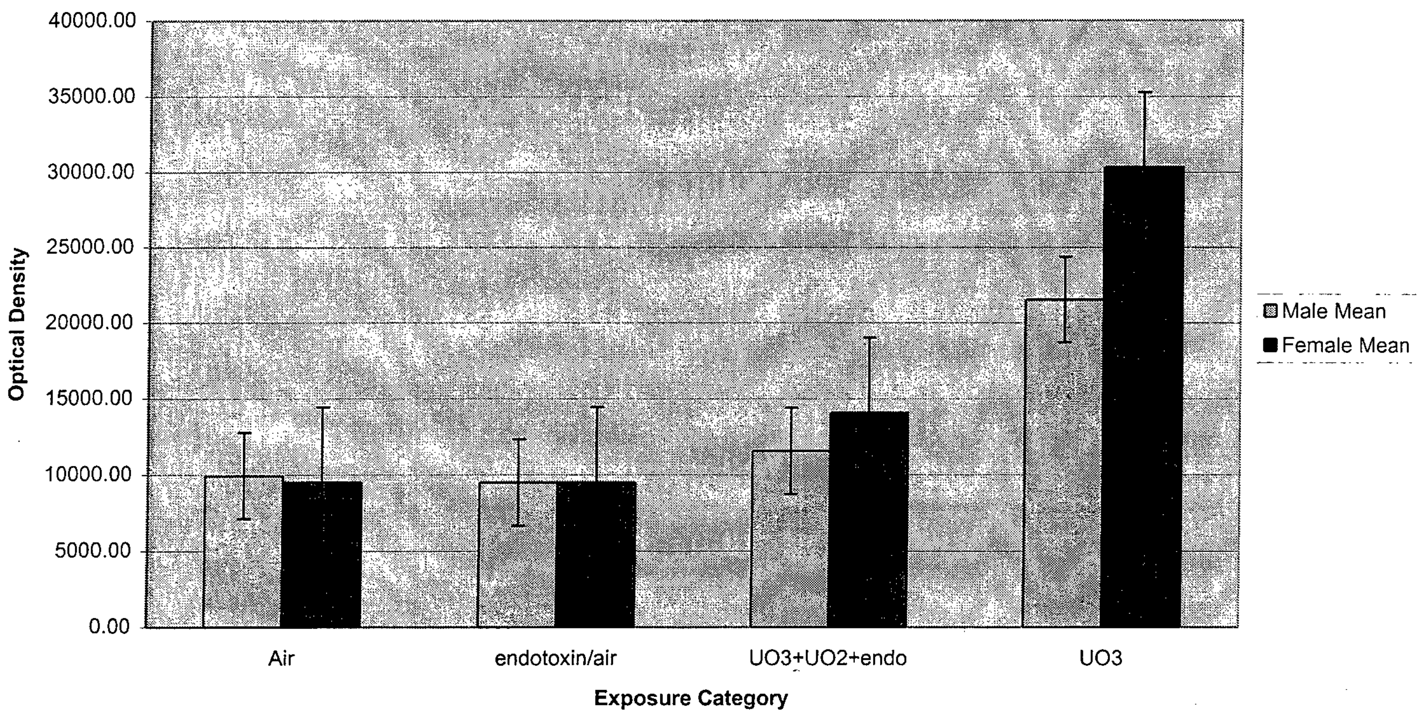

Figure 3. GFAP Density by Gender

KEY RESEARCH ACCOMPLISHMENTS

- Generation of aerosol atmospheres of ~500 mg/m^3 with a mass median aerosol diameter of ~2 μm for TaO_5, UO_3, UO_2, UO_3+UO_2, and DU.
- Exposures of rats for 15 minutes to high concentrations (~500mg/m^3) of these controlled aerosols.

- Identification of UO_3 as acutely toxic via nephrotoxicity under these conditions, and that this effect was most prominent in female animals.
- *Preliminary* findings indicating that an increase in astroglia immunoreactivity to GFAP is observed within the olfactory glomeruli as early as 2 hours following exposure. This response is generally indicative of a protective neuroinflammatory or neuroimmune response to insult of the proximal neurons. This response has been observed in the primary CNS region receiving the primary projections from the olfactory receptor neurons. Again, the response is greater in female than male animals.
- The response of the astroglia, based on the very limited data evaluated to date, is associated with the solubility of the exposure aerosol of uranium, and appears to be stronger in female than male animals. This effect needs to be examined with the broader treatment conditions not yet analyzed.

REPORTABLE OUTCOMES

Because the majority of this year's effort was taken up in generating the aerosols, data from the initial exposures are at this point are too preliminary to publish, present, or utilize to obtain additional research support

CONCLUSIONS

Our ability to achieve aerosols at the target concentrations of 500 mg/m^3 demonstrates that we will be technically able to address our key hypotheses as originally proposed. The ability to include exposures to depleted uranium we feel extends our proposed scope and makes the interpretation of our data more appropriate to the question of Gulf War exposures. Without inclusion of DU aerosols, results would require extrapolation to the DU situation. Although generation of the aerosols required a bit longer time and effort than originally planned, we have been able to accomplish the major tasks identified for Year 1.

We have only begun to analyze the neurochemistry, but the observations of increased glial immunoreactivity following acute exposure to the soluble UO_3 and the preliminary trend observed indicates that the olfactory pathway and subsequent neuronal responses are likely to occur in response to uranium inhalation exposures. The data currently analyzed for uptake are from a sacrifice only 2 hours post 15-minute exposures. This short survival time should have the lowest probability of showing actual uptake. We originally thought uptake would be most likely to be observed at 30 days post-exposure. Those tissues will be analyzed this month. We have encountered some difficulties in antibody reactivity in these studies, and have had to adjust protocols and suppliers. However, nothing beyond routine difficulties in switching protocols from one laboratory to another has been encountered and to date nothing has produced a substantial time lag.

We have had some down-time while the stage-manipulator for the micro-PIXE is being repaired. We were able to analyze all 0-day sacrifice tissues prior to initiating those repairs. That work is in progress and we anticipate being back on-line to analyze the 30 day tissues within two weeks.

We feel data are too preliminary for much interpretation at this point, and far too incomplete. We hope to have several more of the neurochemical markers analyzed in the 0 and 30 day sacrifice groups by the December 2002 project review. Those data should allow us to more fully interpret the response to the acute exposures and suggest any changes in approach that are indicated.

Based on the preliminary results, we suggest continuing with the tasks as approved in the original scope of work is warranted at this time. The relationship observed between the response of the astroglia and the solubility of the exposure atmosphere suggest that important data on the neurotoxicity of uranium will result from the analysis of the full data set of even the 0-day sacrifice following a 15- min high dose exposure. And finally, the increased sensitivity of female rats to both the acute nephrotoxicity and the glial responsiviness suggest gender differences should be a focus of our future analyses.

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